



# Sexually biased transcripts at early embryonic stages of the silkworm depend on the sex chromosome constitution



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## ABSTRACT

In the silkworm, *Bombyx mori*, females are heterogametic (WZ) whereas males have two Z chromosomes. Femaleness of *B. mori* is determined by the presence of the W chromosome, suggesting that there is a dominant feminizing gene on this chromosome. Recently, by transcriptome analysis of *B. mori* embryos, we discovered that a single W-chromosome-derived PIWI-interacting RNA (piRNA) is the long-sought primary determinant of femaleness in *B. mori*. However, sexual bias in the transcriptome of *B. mori* early embryos has not yet been well characterized. Using deep sequencing data from molecularly sexed RNA of *B. mori* embryos, we identified and characterized 157 transcripts that are statistically differentially expressed between male and female early embryos. Most of the female-biased transcripts were transposons or repeat sequences that are produced presumably from the W chromosome. Bioinformatic analysis revealed that these repetitive sequences are piRNA precursors. In contrast, male-biased genes were frequently transcribed from the Z chromosome, suggesting that dosage compensation in Z-linked genes does not occur or is incomplete at early embryonic stages. Our analysis has drawn a picture of a global landscape of sexually biased transcriptome during early *B. mori* embryogenesis and has suggested for the first time that most sexually biased embryonic transcripts depend on sex chromosomes.

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## 1. Introduction

In the silkworm, *Bombyx mori*, females have WZ sex chromosomes and males have ZZ sex chromosomes (Tanaka, 1916). Previous studies clearly showed that *B. mori* femaleness is strongly determined by the presence of the W chromosome irrespective of the Z chromosome number (Fujii and Shimada, 2007). This finding strongly suggests that a dominant feminizing gene *Feminizer* (*Fem*), is present on the W chromosome (Hasimoto, 1933). Despite many efforts, *Fem* has not been identified at the molecular level for more than 80 years. The identification of *Fem* by a classical positional cloning strategy has been hampered by the lack of crossing over in silkworm females. Furthermore, *B. mori* W chromosome is almost completely occupied by nested transposable elements (Abe et al., 2005), which preclude the building of long accurate sequence scaffolds of this chromosome. Using *B. mori* mutant strains that each possess a unique W chromosome structure (Abe et al., 2008), we found that the sex-determining region of the W chromosome

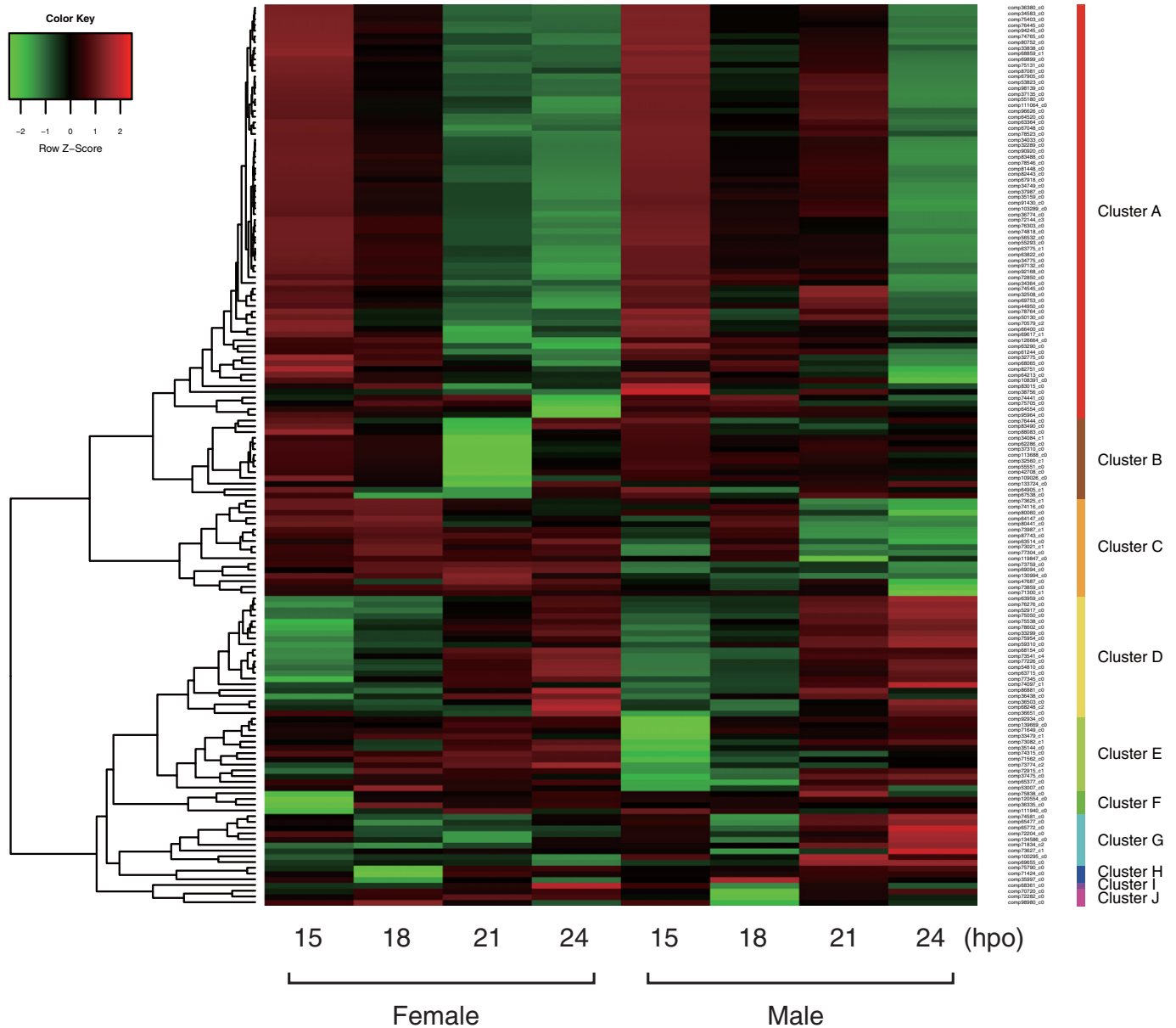
produces female-enriched PIWI-interacting RNAs (piRNAs) that are derived from transposons or repetitive sequences (Kawaoka et al., 2011a). However, the role of these piRNAs in silkworm sexual dimorphisms was unknown until quite recently.

In 2014, we performed deep sequencing (RNA-seq) of molecularly sexed RNA from *B. mori* early embryos and identified 157 transcripts that are statistically differentially expressed between female and male embryos (Kiuchi et al., 2014). Among them, we found one transcript that is highly expressed in females at all stages of embryos, but not in males. This transcript was expressed from the W chromosome and found to be a precursor of a female-specific piRNA. Injection of an RNA inhibitor for this piRNA into female embryos resulted in the production of the male-type splicing of *B. mori doublesex* (*Bmdsx*), a gene acting at the terminal end of the sex differentiation cascade (Suzuki et al., 2003; Suzuki et al., 2005). Considering these results, we concluded that this piRNA precursor is the long-sought feminizing factor and named this RNA *Fem* (Kiuchi et al., 2014). Further experiments identified the target gene of *Fem*-derived piRNA (*Fem* piRNA), which is located on the Z chromosome. Depletion of this Z-linked gene in male embryos led to the production of the female-type splicing of *Bmdsx*, indicating that the product of this gene is a masculinizing factor. We accordingly named the gene *Masculinizer* (*Masc*). Collectively, *B. mori* sex is determined by the *Fem* piRNA-*Masc* cascade (Kiuchi et al., 2014).

Abbreviations: piRNA, PIWI-interacting RNA; hpo, hours post-oviposition; TPM, transcripts per million; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

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**Fig. 1.** Hierarchical cluster analysis of 157 selected genes. Temporal expression profiles of 157 genes exhibiting statistical differences between male and female embryos were hierarchically clustered and visualized in a heatmap. RNA-seq experiments for each time point are in columns; individual genes are in rows. The degree of color saturation reflects the magnitude of the ratio (see color key at the top).

Although our RNA-seq approach identified the primary determinant of *B. mori* sex, we have not completely characterized sexually biased transcripts expressed during early embryogenesis. In this study, we first performed clustering analysis of sexually biased transcripts and divided them into 10 groups according to their expression patterns. Next we characterized each group by accurate annotation of each transcript. The results demonstrate that sexually biased gene expression in *B. mori* early embryos is mainly dependent on the sex chromosome constitution.

## 2. Materials and methods

### 2.1. RNA-seq data

RNA-seq data were processed as previously described in detail (Kiuchi et al., 2014). In brief, *de novo* assembly of RNA-seq data from 8 data sets [15, 18, 21, and 24 h post-oviposition (hpo) of each sex; 303,483,056 reads in total (The accession number DRA001104 in DDBJ)] was performed using Trinity (Grabherr et al., 2011). Transcript

abundance in each transcript was quantified by RSEM (Li and Dewey, 2011). Sexually biased transcripts (adjusted *p*-value < 0.05) were identified with the R/Bioconductor package, DESeq (Anders and Huber, 2010). 157 transcripts with more than 10 transcripts per million (TPM) in any data set were finally selected and used in our analysis.

### 2.2. piRNA mapping

Mapping of embryonic piRNAs (Kawaoka et al., 2011b) was performed with Bowtie (Langmead et al., 2009) allowing no mismatch as described previously (Kiuchi et al., 2014). Ping-pong significance was evaluated as described previously (Kawaoka et al., 2011a).

### 2.3. Mapping of RNA-seq data onto *B. mori* gene models

RNA-seq data (The accession numbers DRA001104 and DRA001338 in DDBJ) (Kiuchi et al., 2014) were mapped onto 14,623 *B. mori* gene models (putative protein-coding genes) with TopHat2 (Kim et al.,

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